Synthesis of New Heparinoids with High Anticoagulant Activity

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New heparinoids were synthesized by the chemical method starting from ring-opening polymerization of anhydro sugar derivatives. Sulfation of synthetic (1→6)-α-linked 3-amino-3-deoxy-D-glucopyranan and its copolymers gave dextran-type heparinoids having a sulfamide group on the C-3 carbon of the sugar unit. Heparinoids with different sulfamide contents indicated that the anticoagulant activity (35.3–41.3 units/mg) is independent of the sulfamide content, while an increase in sulfamide content lowered the toxicity. Sulfation of (1→5)-α-D-xylofuranan and -ribofuranan provided furanan-type heparinoids the anticoagulant activities of which were higher than those of the corresponding sulfated pyranan-type polysaccharides (1→4)-β-D-xylopyranan and -ribopyranan. The highest activity (69.1 units/mg) was shown by sulfated (1→5)-α-D-xylofuranan. The dextran-type heparinoid having a sulfamide group showed a high anticoagulant activity also in vivo and high lipemia-clearing activity.

Since the discovery of heparin and its biological activities, the structure of the site necessary for anticoagulant activity was investigated and found to be composed of a pentasaccharide residue consisting of D-glucosamine, D-glucuronic acid, and L-iduronic acid, the hydroxyl and amino groups of which are partly sulfated and to a smaller extent acetylated.[1]

The high anticoagulant activity of heparin can be attributed to the synergism of *N*-sulfonic acid and *O*-sulfonic acid, both of which are in the form of sodium salt, and other factors including the configuration of the polysaccharide. Thus, when *O*-sulfonic acid groups were removed, anticoagulant activity was dramatically lowered.[2] In addition, when chitosan[3] having amino groups and chitin[4] having acetamido groups were sulfated, these heparinoids showed low activities.

Several investigations have reported on the synthesis of heparin fragments. Recently, Sinaÿ and co-workers synthesized the heparin pentasaccharide fragment which showed strong binding with antithrombin III and induced high anti-Xa activity.[5]

To synthesize heparinoids, the sulfation of natural polysaccharides[6-8] and artificial pseudopolysaccharides[9] was performed by various methods. Lately, Muzzarelli et al. reported the synthesis of sulfated *N*-(carboxymethyl)-chitosans, which show fairly high anticoagulant activity.[10]

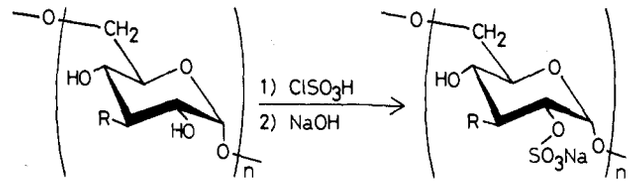
Low-molecular-weight dextran sulfate, which has low anticoagulant activity, is used clinically as an antithrombogenic substance. Thus, to increase the anticoagulant activity of the polysaccharides, we synthesized a dextran having amino groups, which will be changed into sulfamide groups by sulfation.[11,12]

In this paper, we report the first synthesis of sulfated 3-amino-3-deoxy-(1→6)-α-D-glucopyranan that contains sulfamide and *O*-sulfonic acid groups. Since it is not known which factor in the blood coagulation system reacts with a new heparinoid, the anticoagulant activity in vitro is determined with bovine plasma by a modification of United States Pharmacopoeia. We also report the synthesis of sulfated (1→5)-α-D-xylofuranan and -ribofuranan, which have high anticoagulant activities. In addition, the anticoagulant activity of sulfated (1→4)-β-D-xylopyranan (natural xylan) and -ribopyranan is compared with that of the sulfated furanan-type polysaccharides.

Chemistry

1. Dextran-Type Polysaccharide with Sulfamide Group. In an approach toward the regioselective synthesis of aminated polysaccharides, the cationic ring-opening polymerization and copolymerization of 1,6-anhydro-β-D-glucopyranose derivatives having an azido group was studied.[11,12] Reduction of benzylated (1→6)-α-D-glucopyranan having different amounts of azido groups with lithium aluminum hydride and subsequent debenzylation gave amino-group-containing (1→6)-α-D-glucopyranan (**1**, **2**).

Scheme I



1 R = NH_2
2 R = NH_2 or OH
3 R = $NHSO_3Na$
4 R = $NHSO_3Na$ or OSO_3Na

Sulfation of the polysaccharides **1** and **2** (Scheme I) was carried out with chlorosulfuric acid in pyridine according to a modification of the method of Wolfrom and Shen Han.[13] The sodium salt of the resulting polymer (**3**, **4**) was soluble in water. The IR spectrum of the sulfated polymer indicated the existence of broad absorptions due to *N*-sulfonic acid or *O*-sulfonic acid. Moreover, a negative

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Table I. Anticoagulant Activity in Vitro and LD_{50} of Sulfated (1→6)-α-Glucans

polymer	amino content[a]	sulfur content, %	$\bar{M}_n \times 10^{-4}$ [b]	anticoag act. (AA), units/mg	LD_{50}, mg/kg	AA × LD_{50}
3	1.0	16.1	1.45	35.3	1320	46 600
4	0.5	14.7	2.01	41.3	710	29 300
NC-1020[c]	0.0	18.4	0.58	19.3	2500	48 300

[a] Number of amino groups per sugar unit. [b] Determined by gel permeation chromatography. [c] Commercial dextran sulfate (Meito Sangyo).

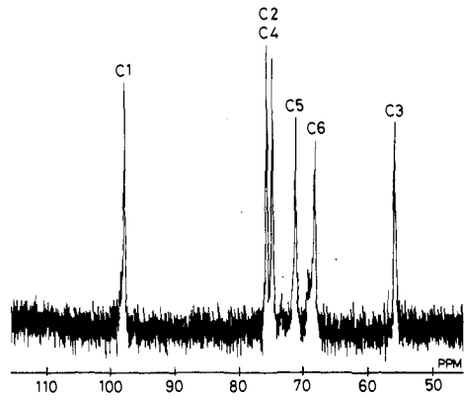


Figure 1. ^{13}C NMR (100-MHz) spectrum of 3-amino-3-deoxy-(1→6)-α-D-glucopyranan sodium sulfonate (**3**) (D_2O solvent).

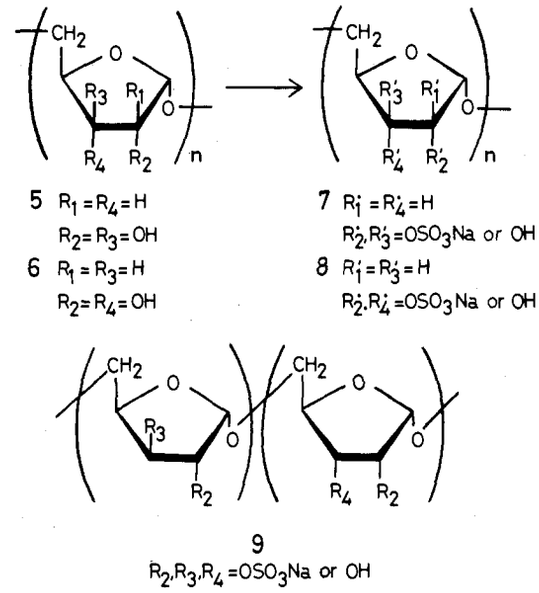
ninhydrin reaction of the obtained polymer exhibited the absence of free amino groups, and the metachromasia reaction was positive, indicating the existence of sulfonate. These data indicated that the amino group in the polysaccharide was completely sulfated.

As shown in Figure 1, the ^{13}C NMR spectrum of the sulfated 3-amino-3-deoxy-(1→6)-α-D-glucopyranan **3** shows that the structure of the polymer is highly stereoregular. Elemental analysis of **3** indicates that the polymer has two sulfur atoms/3-amino-3-deoxyglucose unit and contains a small amount of water. Since it is known that the order of reactivity of the secondary hydroxyl groups of dextran is 2-OH > 3-OH > 4-OH in partial sulfation,[14] it is assumed that the 2-hydroxyl and 3-amino groups were sulfated in this polymer.

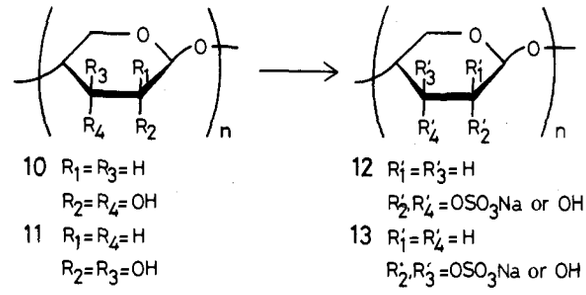
2. Sulfated (1→5)-α-D-Pentofuranans and (1→4)-β-D-Pentopyranans. (1→5)-α-D-Xylofuranan (**5**), which has the backbone structure containing the five-membered furanose ring, was synthesized by the selective 1,5-ring-opening polymerization of 1,4-anhydro-2,3-di-*O*-benzyl-α-D-xylopyranose (= 1,5-anhydro-2,3-di-*O*-benzyl-β-D-xylofuranose) with boron trifluoride etherate as catalyst at −60 °C to 2,3-di-*O*-benzyl-(1→5)-α-D-xylofuranan and by subsequent debenzylation.[15] In the same manner, (1→5)-α-D-ribofuranan (**6**) was synthesized by the selective ring-opening polymerization of 1,4-anhydro-2,3-di-*O*-benzyl-α-D-ribopyranose and by subsequent debenzylation.[16] Since the α-furanosidic linkages of **5** and **6** are very sensitive to acidic conditions, the nondegrading sulfation of **5** and **6** was carried out with piperidine-*N*-sulfonic acid in a homogeneous dimethyl sulfoxide solution (Scheme II). The sulfated xylofuranan **7** has trans substituents at C-2 and C-3 carbons, and the sulfated ribofuranan **8** has cis substituents.

(1-5)-α-Linked heteropolysaccharide containing 66% α-D-xylofuranosidic and 34% α-D-ribofuranosidic units was treated to give a sulfated (1→5)-α-riboxylan (**9**). The α-stereoregularity and the copolymer composition of the heteropolysaccharide were determined by ^{13}C NMR spectroscopy.

Scheme II



Scheme III



Metachromasia reaction of **7**, **8**, and **9** was positive, showing that the sulfation had occurred. The elemental analysis of the polymers suggested that the number of sulfur atoms/sugar unit in the polymers **7**, **8**, and **9** was ca. 1.4–1.7.

To examine the relationship between polymer structure and anticoagulant activity, stereoregular polypentoses having six-membered pyranose rings were sulfated (Scheme III). A highly stereoregular (1→4)-β-D-ribopyranan (**10**) was prepared by the selective 1,4-ring-opening polymerization of 1,4-anhydro-2,3-*O*-benzylidene-α-D-ribopyranose with antimony pentachloride as catalyst and by subsequent debenzylidenation.[17] The resulting polymer was sulfated with chlorosulfuric acid to give a sulfated ribopyranan (**12**). On the other hand, since (1→4)-β-D-xylopyranan cannot be obtained by any synthetic method, wood xylan, which is a (1→4)-β-D-xylopyranan, was extracted from wood pulp according to the method of Matsuzaki et al.[18] The xylopyranan was also

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Table II. Lipemia-Clearing Activity of Heparinoids in Vivo

heparinoid	dose, mg/kg	lipemia-clearing act.: (Δ(−log T)) before admin	10 min after admin
3	5	0.016	0.775
4	5	0.019	0.764
dextran sulfate[a]	5	0.017	0.566

[a] Commercial dextran sulfate (Meito Sangyo, NC-1020).

sulfated with chlorosulfuric acid to provide a sulfated (1→4)-β-D-xylopyranan (**13**).

Biological Results and Discussion

The dextran-type heparinoids were evaluated for anticoagulant activity in vitro, which was determined by using bovine plasma according to a modification of the United States Pharmacopoeia. The results are summarized in Table I.

Sulfated (1→6)-α-D-glucopyranans with different sulfamide contents showed anticoagulant activity of 35.3–41.3 units/mg, which was about 1.8–2.1 times as high as that of a commercial dextran sulfate (19.3 units/mg). It is noteworthy that the in vitro values of anticoagulant activity for the two kinds of heparinoids were similar and almost independent of the sulfamide content. That is, the introduction of sulfamide groups did not cause an increase in anticoagulant activity.

It is known that the anticoagulant activity of dextran sulfate increases with increasing molecular weight of the polysaccharide. However, the dextran sulfate with high molecular weight is toxic.[19] Therefore, the toxicity of the artificial polysaccharides was tested and the results are shown in Table I. The sulfated glucan with a higher sulfamide content showed a marked decrease in toxicity. Thus, we calculated the value of anticoagulant activity times the LD_{50}, that is, the anticoagulant activity including toxicity. Among the synthetic heparinoids, polymer **3**, which contained one sulfamide group/sugar unit, showed the highest value. Therefore, it was revealed that the sulfamide group works more effectively as the anticoagulant group by decreasing the toxicity than the *O*-sulfate group.

For sulfated (1→6)-α-D-glucopyranans containing sulfamide groups, the anticoagulant activity in vivo was also tested with rabbits. The clotting time was measured for the blood, which was taken from a vein of the rabbit 10 min after intravenous injection of the polymer (25 mg/kg). In any case, the clotting time was more than 60 min, while the clotting time in the control test was less than 10 min. As a result, it was confirmed that the synthetic heparinoids show anticoagulant activity in vivo.

Lipemia-clearing activity of the artificial heparinoids was tested on rabbits according to the Hara–Kuzuya method.[20] The difference in absorbance (Δ(−log *T*)) between plasma taken from a vein of the rabbit 10 min after an intravenous injection of the heparinoid (5 mg/kg) and the plasma after incubation for 3 h was determined. The results are shown in Table II. Synthetic heparinoids showed a high lipemia-clearing activity that was 1.4 times as high as that of the commercial dextran sulfate.

Since it was found that sulfamide groups were not essential to get a heparinoid with high anticoagulant activity and a polysaccharide substituted regioselectively by sulfate groups is expected to exhibit a high activity, the synthesis of new heparinoids was tried by sulfation of stereoregular polypentoses newly synthesized in our laboratory.[15–17]

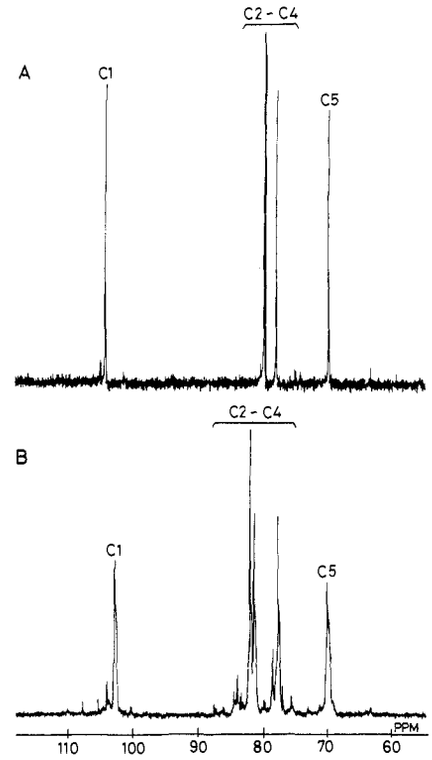

Figure 2. ^{13}C NMR (67.8-MHz) spectra of (A) (1→5)-α-D-xylofuranan (**5**) and (B) sulfated (1→5)-α-D-xylofuranan (**7**) (D_2O solvent).

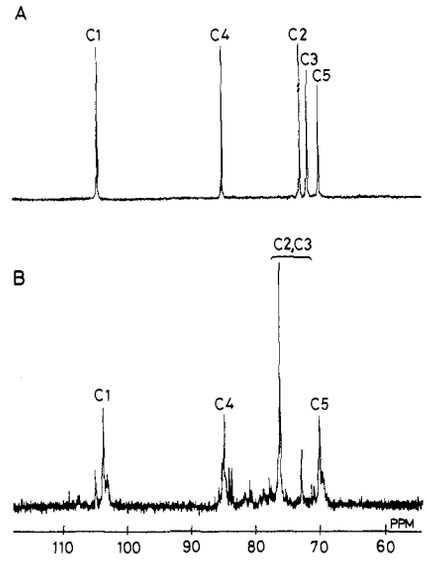

Figure 3. ^{13}C NMR (67.8-MHz) spectra of (A) (1→5)-α-D-ribofuranan (**6**) and (B) sulfated (1→5)-α-D-ribofuranan (**8**) (D_2O solvent).

The ^{13}C NMR spectra show that incomplete sulfation of the two hydroxyls occurred, though the original (1→5)-α-linked polypentoses were highly stereoregular (Figures 2 and 3).

The sulfated (1→5)-α-D-pentofuranans and (1→4)-β-D-pentopyranans were evaluated for anticoagulant activity in vitro with bovine plasma by using the same method as that used for the dextran-type heparinoids. The results are summarized in Table III. The anticoagulant activity (69.1 units/mg) of the sulfated xylofuranan **7** was 1.5 times

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Table III. Anticoagulant Activity in Vitro of Sulfated (1→5)-α-D-Pentofuranans and (1→4)-β-D-Pentopyranans

sulfated polymer	backbone structure	sulfur content, %	$\bar{M}_n \times 10^{-4}$ [a]	anticoag act., [b] units/mg
7	(1→5)-α-D-xylofuranose	16.1	1.24	69.1
8	(1→5)-α-D-ribofuranose	15.5	0.89	56.0
9	(1→5)-α-D-xylofuranose (66%) + (1→5)-α-D-ribofuranose (34%)	15.2	0.86	23.7
12	(1→4)-β-D-ribopyranose	15.1	3.48	41.2
13	(1→4)-β-D-xylopyranose	15.1	1.29	45.5

[a] Determined by gel permeation chromatography. [b] Commercial dextran sulfate (Meito Sangyo, NC-1020), 19.3 units/mg.

as high as that of the sulfated xylopyranan **13**. Since both sulfated xylofuranan and xylopyranan have similar molecular weights and sulfur contents, the difference in the activity between the two polysaccharides can be ascribed to the difference in polymer configuration. The activity of 69.1 units/mg for the xylofuranan was 3.6 times as high as that of the commercial dextran sulfate (Meito Sangyo Co., Ltd., NC-1020) and about 40% of the activity of a natural heparin.

The anticoagulant activity (56.0 units/mg) of the sulfated ribofuranan **8** was higher than that (41.2 units/mg) of the sulfated ribopyranan **12**, although the molecular weight of the former was about a quarter of that of the latter. Thus, it was revealed that the synthetic furanan-type heparinoids show higher anticoagulant activities than the pyranan-type heparinoids.

Recently, the effects of heparin and synthetic heparinoids on several blood-coagulant factors were studied. The anticoagulant activity of heparin is caused by its complexing with antithrombin III, which accelerates the formation of a stable 1:1 complex between antithrombin III and thrombin.[21] It has been shown that heparin cofactor II also has a function similar to antithrombin III in the presence of heparin.[22] Oshima et al. reported that dextran sulfate does not bind to antithrombin III and strongly binds to thrombin probably at a site other than the active site of thrombin.[23] Hirano et al. reported that sulfated chitosan derivatives show high anticoagulant activities with respect to activated partial thromboplastin time and have very low activities with respect to antithrombin activity.[24] These investigations suggest that the synthetic heparinoids work as anticoagulants in a mechanism different from that of heparin.

Since it is known that the polymer backbone of furanan-type polysaccharides is more flexible than that of the pyranan-type polysaccharides, such flexibility of the polymer backbone might be one of the reasons why the furanan-type heparinoids exhibit higher activities, that is, the flexible polymer backbone of the heparinoid might be favorable for interacting nonspecifically with blood-coagulant-factor polymers. In addition, the high activity may be caused by a regular structure of the polysaccharide, because the sulfated copolysaccharide (1→5)-α-riboxylan **9** had a lower activity (23.7 units/mg) than both (1→5)-α-ribofuranan and -xylofuranan. The function of the synthetic heparinoids on respective blood-coagulant factors is now being investigated.

Experimental Section

^{13}C NMR spectra, 100 and 67.8 MHz, were recorded on JEOL GX-400 and GX-270 spectrometers, respectively, in deuterium oxide with 4,4-dimethyl-4-silapentane sodium sulfonate (DSS) as internal standard. The IR spectrum was measured by means of a JASCO IRA-2 spectrometer. The number-average molecular weight ($\bar{M}_n$) of sulfated polysaccharides was determined by aqueous-phase gel permeation chromatography (columns, Toyo Soda TSK-gel; eluent, 66.7 mM phosphate buffer, pH 6.86) using standard dextran as reference. Sulfur content analysis was performed by Riken (The Institute of Physical and Chemical Research) analytical laboratories.

3-Amino-3-deoxy-(1→6)-α-D-glucopyranan Sodium Sulfonate (3). Sulfation of the aminoglucan with chlorosulfuric acid was carried out according to a modification of the method of Wolfrom and Shen Han.[13] 3-Amino-3-deoxy-(1→6)-α-D-glucopyranan (**1**)[11] (100 mg) was suspended in water and collected by centrifugation. The white precipitate was successively washed with ethanol three times and with ethyl ether three times and finally suspended in 8 mL of pyridine. To a chlorosulfuric acid (1 mL) solution in pyridine was added the aminoglucan suspension in pyridine, and the mixture was heated on a boiling-water bath for 1 h. After cooling, the mixture was poured into 20 mL of water, and then 7.5 mL of 2.5 N NaOH was added. The yellowish solution was dialyzed with running water for 3 days in a cellulose tube. After the solution was concentrated under reduced pressure, the sulfated polymer **3** was freeze-dried from water: yield, 183.7 mg; $[\alpha]^{25}_D$ +116.9° (c 1, water); ninhydrin, −; metachromasia, +; $\bar{M}_n = 1.45 \times 10^4$; IR (KBr) 580, 610, and 1240 cm^{-1}; ^{13}C NMR (D_2O) 97.53 (C-1), 75.54 and 74.68 (C-2, C-4), 71.03 (C-5), 68.07 (C-6), and 55.75 ppm (C-3) (spectrum shown in Figure 1). A (1→6)-α-linked heteropolysaccharide consisting of 50% 3-amino-3-deoxy-D-glucopyranosidic units (**2**)[12] (100 mg) was sulfated in the same manner to give **4**: yield, 168.0 mg; $[\alpha]^{25}_D$ +100.2° (c 1, water) [η] 0.07 (H_2O, 30 °C).

(1→5)-α-D-Pentofuranan Sodium Sulfonates 7, 8, and 9. Sulfations of (1→5)-α-D-pentofuranans were carried out according to the method of Nagasawa and Yoshidome.[25] To 100 mg of (1→5)-α-D-xylofuranan (**5**), -ribofuranan (**6**), or their copolysaccharide solution in 36 mL of dimethyl sulfoxide was added piperidine-*N*-sulfonic acid (600 mg). Each polysaccharide had been treated with 100 mg of sodium borohydride in 50 mL of water. The reaction mixture was heated for 1 h at 80 °C with stirring, and then it was diluted with saturated sodium bicarbonate solution. The yellowish solution was dialyzed with running saturated sodium bicarbonate solution for 1 day and then with running distilled water for 3 days in a cellulose tube. After the solution was concentrated under reduced pressure, the sulfated pentofuranan (**7**, **8**, or **9**) was freeze-dried from water: yield, 83–139 mg; metachromasia, +. Sulfur contents and number-average molecular weights of the sulfated polymers are shown in Table III.

(1→4)-β-D-Pentopyranan Sodium Sulfonates 12 and 13. Sulfations of (1→4)-β-D-pentopyranans were carried out according to a modification of the method of Wolfrom and Shen Han.[13] To 100 mg of a (1→4)-β-D-pentopyranan (synthetic ribopyranan **10** or natural xylopyranan **11**) suspension in 4 mL of dried pyridine was added chlorosulfuric acid (2 mL) that had been added to 12 mL of the cooled pyridine, and the mixture was heated on a boiling-water bath for 1 h. After dialysis, the sulfated pentopyranan (**12**, **13**) was freeze-dried from water: yield, 97–100 mg; metachromasia, +. Sulfur contents and number-average molecular weights of the sulfated polymers are shown in Table III.

Biological Test Procedures. Anticoagulant activity testing in vitro was performed by use of bovine plasma according to a

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modification of the United States Pharmacopoeia. In each test tube (15 mL), artificial heparinoid was dissolved in 0.8 mL of a physiological saline solution (0.016, 0.014, 0.012, 0.010, 0.008, 0.006, 0.004, 0.002, 0.001 w/v %). Physiological saline solutions of standard dextran sulfate (Meito Sangyo, NC-1020) were also prepared in nine stages of concentration in the same manner as above. To each solution were added 1.0 mL of bovine plasma that had been pretreated with sodium citrate and 0.2 mL of aqueous calcium chloride (2 w/v %), and then the mixture was allowed to stand for about 15 min. The state of coagulation in each test tube was evaluated with five steps, which were 0.0, 0.25, 0.5, 0.75, and 1.0. Concentration of the artificial heparinoid (Ca) and the standard dextran sulfate (Cs) showing the coagulation-state value of 0.5 were calculated by using logarithm. Then, the anticoagulant activity of the artificial heparinoid (Aa) was calculated according to the equation Aa = As × Cs/Ca, where As was anticoagulant activity of the standard dextran sulfate.

Acknowledgment. We thank Meito Sangyo Co., Ltd. for helpful cooperation in the physiological activity tests and for supply of NC-1020 dextran sulfate. We are grateful to Dr. Alan D. Elbein for his helpful advice.

Registry No. **1**, 85553-36-2; **3**, 107037-81-0; **5**, 105471-72-5; **6**, 87481-53-6; **7**, 107037-84-3; **8**, 107037-87-6; **10**, 87481-54-7; **11**, 107037-88-7; **12**, 107037-91-2; **13**, 107037-94-5.

Synthesis and Neuroleptic Activity of a Series of 1-[1-(Benzo-1,4-dioxan-2-ylmethyl)-4-piperidinyl]benzimidazolone Derivatives

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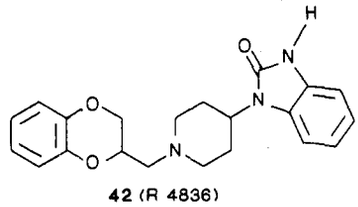
A series of 1-[1-(benzo-1,4-dioxan-2-ylmethyl)-4-piperidinyl]benzimidazolones with various substituents in both aromatic rings have been synthesized and tested for neuroleptic activity (antiapomorphine effects and [^{3}H]spiroperidol binding) as well as extrapyramidal effects (cataleptogenic effect). A strong dependence of activity on the 5-substituent in the benzimidazolone moiety could be demonstrated. Some compounds show a large split between the desired antiapomorphine and the undesired extrapyramidal effect. From these, 1-[1-(benzo-1,4-dioxan-2-ylmethyl)-4-piperidinyl]-5-chlorobenzimidazol-2-one hydrochloride (HR 723), **12**, has been selected for further preclinical and toxicological profiling.

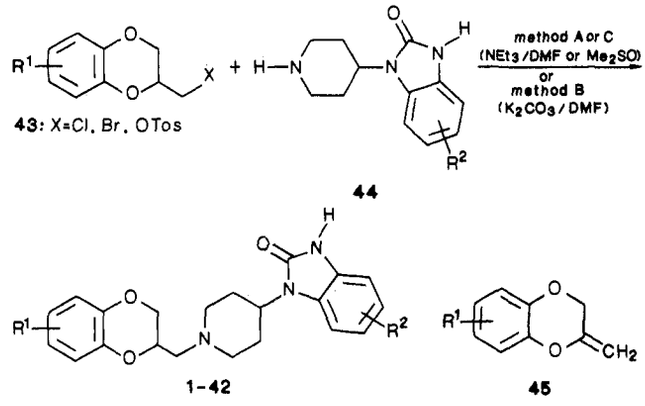
Most neuroleptic drugs that are in use in the clinical treatment of the symptoms of schizophrenia to date (e.g., butyrophenones like haloperidol and phenothiazines like chlorpromazine)[1] are plagued with the appearance of undesired and sometimes irreversible side effects, most prominently parkinson-like symptoms and tardive dyskinesia,[2] resulting from interference of these agents with dopamine receptors in the extrapyramidal system of the brain (EPS symptoms).[3] We became interested in designing potent neuroleptic agents that are less prone to the induction of these side effects. On the basis of this objective, we have designed and prepared a series of compounds containing benzo-1,4-dioxanyl and (4-piperidinyl)benzimidazolone moieties. The fully unsubstituted compound (**42**) (R 4836) has been shown to possess some neuroleptic activity.[4] Furthermore, a (4-



42 (R 4836)

piperidinyl)benzimidazolone unit occurs in potent neuroleptics such as benperidol,[5] pimozide,[6] and halopemid[7] and

Scheme I



analgesics like benzitramid.[8]

The prepared compounds were subjected to screening tests for antiapomorphine activity and biochemical testing on brain dopamine receptors.[9] Activity in these tests is indicative for the clinical potency of neuroleptic drugs in schizophrenic patients.[10] Although the propensity to in-

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0022-2623/87/1830-0814$01.50/0 © 1987 American Chemical Society